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Swelling-induced taurine release without chloride channel activity in *Xenopus laevis* oocytes expressing anion channels and transporters

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Abstract

Taurine is an important osmolyte involved in cell volume regulation. During regulatory volume decrease it is released via a volume-sensitive organic osmolyte/anion channel. Several molecules have been suggested as candidates for osmolyte release. In this study, we chose three of these, namely ClC-2, ClC-3 and I_{Cln}, because of their expression in rat astrocytes, a cell type which is known to release taurine under hypotonic stress, and their activation by hypotonic shock. As all three candidates were also suggested to be chloride channels, we investigated their permeability for both chloride and taurine under isotonic and hypotonic conditions using the *Xenopus laevis* oocyte expression system. We found a volume-sensitive increase of chloride permeability in ClC-2-expressing oocytes only. Yet, the taurine permeability was significantly increased under hypotonic conditions in oocytes expressing any of the tested candidates. Further experiments confirmed that the detected taurine efflux does not represent unspecific leakage. These results suggest that ClC-2, ClC-3 and I_{Cln} either participate in taurine transport themselves or upregulate an endogenous oocyte osmolyte channel. In either case, the taurine efflux of oocytes not being accompanied by an increased chloride flux suggests that taurine and chloride can be released via two separate pathways.

Keywords: Regulatory volume decrease; Osmolyte; Taurine; Efflux; Chloride channel; Astrocyte

1. Introduction

In many cell types, swelling stimulates the release of inorganic and organic solutes, which results in a regulatory volume decrease (RVD). Taurine is one of the major osmolytes in mammalian cells and is also released during RVD. Release of taurine has been studied, among others, in glial cells [1,2], erythrocytes from different species [3], HeLa cells [4,5], 9HTEo human tracheal cells [6], and endothelial cells [7].

The mechanism of taurine release shares a number of characteristics with swelling-induced anion channels. Both, the release of chloride and the release of taurine follow the same time course and both efflux pathways can be inhibited by anion channel blockers like NPPB, DIDS, tamoxifen and niflumic acid [1,2]. In whole cell patch clamp experiments, a significant permeability of swelling-induced anion channels for taurine, aspartate and glutamate could be demon-

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strated [8]. In contrast, in other cell types, swelling-induced taurine release and swelling-induced chloride channels appear to have different pharmacological profiles [5,9]. Taurine release has been intensively investigated in cultured astrocytes [1,10]. In most mammalian cells, regulatory volume decrease is mainly achieved by release of K^+ and Cl^-. Yet, in the brain, organic osmolytes are especially important in volume regulation as they do not interfere with the K^+-homeostasis of the brain, which is crucial for neurotransmission. Volume regulation by releasing organic osmolytes is, therefore, a mechanism which might be particularly important for the function of astrocytes.

A number of molecular candidates have been suggested to be involved in regulatory volume decrease (RVD) by release of anions or osmolytes: e.g. the chloride channel family members ClC-2 [11] and ClC-3 [12], the I_{Cln} protein [13], the anion exchanger Band 3 (AE-1) [14], the mitochondrial voltage-dependent anion channel (VDAC) [15] and the small membrane protein phospholemman [16]. From these proteins, only AE1 has been investigated in respect to taurine permeation. By expression in *X. laevis* oocytes, it could be demonstrated that AE-1 from mouse was not permeable for taurine under hypo-osmotic conditions, whereas the orthologous protein from trout acted as a taurine release pathway under these conditions [14]. The chloride channels ClC-2 and ClC-3 have been described as volume-sensitive chloride channels. When expressed in *Xenopus* oocytes, rat ClC-2 showed an increased Cl^- conductance under hypo-osmotic conditions [11]. ClC-3 first been cloned from rat brain [17] was subsequently cloned from guinea pig heart muscle and shown to act as a volume-sensitive chloride channel when overexpressed in transfected 3T3 cells [12]. Neither ClC-2 nor ClC-3 have been investigated in respect to their permeability for osmolytes. Additionally ClC-3 might form heteromultimers with other channels of the ClC-family, as it was found for ClC-1 and ClC-2 [18].

Expression of the I_{Cln} protein in *Xenopus* oocytes was found to induce outwardly rectifying chloride currents which are similar to the currents elicited in cultured cells under hypo-osmotic conditions [19]. However, its precise structure and function remains controversial. On the one hand, the I_{Cln} protein has been proposed to form a chloride channel itself [13]. Other groups, however, have allocated I_{Cln} protein to the cytosol, where it might be associated with actin. This suggests a regulatory role rather than a channel function of the I_{Cln} protein [19]. A further model proposes I_{Cln} as a mediator between a cytoskeletal volume-sensor and the volume-regulated ClC-3 [20].

Similar to I_{Cln}, the precise cellular location of the voltage-dependent anion channel (VDAC) has not been clarified as well. VDAC is established as a channel of the outer mitochondrial membrane, however its allocation to the plasma membrane remains controversial [21].

In this study, we have investigated the expression of ClC-2, ClC-3 and I_{Cln} in cultured astrocytes which show significant release of taurine under hypo-osmotic conditions. Furthermore, we expressed all three candidates in the *X. laevis* oocytes expression system in order to investigate their permeation properties.

2. Materials and methods

2.1. Materials

L-[1,2-^{3}H]Taurine (703 Gbq/mmol), [^{3}H]inulin (29.6 GBq/mmol) and L-[U-^{14}C]lactate (5.66 Gbq/mmol), ^3H_2O (185 MBq/ml) and $Na^{36}Cl$ (18.6 GBq/mol) were purchased from Amersham Buchler (Braunschweig, Germany). The RNA cap structure analog 7 mG(5′)ppp(5′)G was purchased from New England Biolabs (Schwalbach, Germany). Restriction enzymes, nucleotides and polymerases were from Life Technologies, (Eggenstein, Germany) or Boehringer-Mannheim (Germany), the Taq-polymerase from Life Technologies (Eggenstein, Germany), the Pfu-polymerase was from Promega (Mannheim, Germany). The PCR-Script AMP cloning kit was purchased from Stratagene (Amsterdam, Netherlands). Collagenase (EC 3.4.24.3; 0.3 U/mg from *Clostridium histolyticum*) was purchased from Boehringer-Mannheim (Germany); lots were tested for their suitability for oocyte expression. Streptolysin S was obtained from Sigma (Deisenhofen, Germany). All other chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Boehringer-Mannheim (Germany).

2.2. Methods

2.2.1. Cell culture

Astroglia-rich primary cultures were prepared from newborn rat brains as described before [22]. Briefly, total brains were passed successively through two nylon nets of 211 and 135 μm mesh width. The suspended cells of complete offspring (10–15 animals) were collected by centrifugation and resuspended in 20 ml DMEM/10% FCS. After determination of the cell number, cells were diluted in DMEM/10% FCS to a final density of 200 000 cells/ml. Aliquots were dispensed in cell culture dishes of appropriate size and incubated in a cell incubator at 37°C, 90% air, 10% CO_2 for 14–21 days. The medium was renewed every week. Neuron-rich primary cultures were derived from embryonal rat brains (E16) as described before [22]. Briefly, embryo brains were passed successively through two nylon nets of 135 and 20 μm mesh width. One million cells were seeded onto poly-D-lysine coated 35 mm dishes. After 3 days in culture, cells were treated for 24 h with cytosine arabinoside at a final concentration of 0.5 μM to kill dividing cells. Subsequently, cells were incubated in glia-conditioned neuron culture medium. The cultures were used at a culture age of 5–7 days. These cultures contain some astroglial cells, but no oligodendroglial or ependymal cells as determined by cell-specific markers.

2.2.2. RT-PCR

Total RNA was isolated from astroglia-rich and neuron-rich primary culture and from fetal (E16) and adult rat brain by the acid guanidinium thiocyanate–phenol–chloroform extraction method. mRNA was isolated from total RNA using oligo-dT cellulose. mRNA was reverse transcribed with Superscript II reverse transcriptase using an oligo-dT primer.

For the detection of ClC-2, ClC-3 and I_{Cln} mRNA in rat brain the following primers were used: oligonucleotides ICLNs 5′-AGCTTCCTCAAAAGCTTCCC-3′ and ICLNa 5′-GGTGGGGTACTCCAGTGAGA-3′, corresponding to bases 45–64 and to bases 221–202, respectively, of the rat I_{Cln} cDNA [19] (accession no. L26450) were used to amplify a 177-bp product. The corresponding sequences are 98% identical in rat and canine I_{Cln} cDNA [13]. ClC-3 primers were specific to ClC-3 sequence as to avoid coamplification of other members of the ClC-family. Oligonucleotides ClC3s 5′-CAGCCATTACTGCTGTGATAGCC-3′ and ClC3a 5′-GCTCCACAGCAATCCCCTCAATC-3′ corresponding to bases 1628–1650 and to bases 1963–1941, respectively, of the ClC-3 cDNA sequence [17] (accession no. D17521) were used to amplify a 336-bp product. The ClC-2 primers were chosen to distinguish between the cDNA sequences of two splice variants ClC-2L and ClC-2S which contain or lack the sequence encoded by exon 20 [23] (accession no. AF005720). Oligonucleotide ClC2s 5′-CACAGAGGACTCAGGCTTCC-3′ corresponded to bases 2220–2239 ClC-2 cDNA sequence (encoded by exon 19) and oligonucleotide ClC2a 5′-TCATCTTGCCTTCCAGGTCT-3′ corresponded to bases 2494–2475 of the ClC-2 cDNA (encoded by exon 21). The length of the product depends on the presence (275 bp) or absence (215 bp) of the cDNA sequence of exon 20. For this analytical PCR cDNA was amplified in a 30-cycle PCR reaction (60 s at 94°C–60 s at 55°C–30 s at 72°C) using Taq-polymerase. The identity of the PCR products was verified by restriction analysis. The digestion of the 177-bp product of rat I_{Cln} with *Pst*I yielded fragments of 125 and 52 bp, except for the control, which was amplified from a plasmid containing the canine sequence. The ClC-3 specific PCR product of 336 bp was cut by *Pvu*I into a 305- and a 31-bp fragment. Digestion of the ClC-2 specific products with *Kpn*I yielded in any case a 105-bp fragment and additionally a 110- or a 170-bp fragment resulting from the short (215 bp) or the long product (275 bp), respectively.

2.2.3. Cloning of ClC-3

Rat ClC-3 cDNA was cloned by high fidelity RT-PCR. For this, mRNA isolated from astroglia-rich primary cultures, was reverse transcribed. For PCR, two primers were constructed which flanked the coding sequence of ClC-3. Oligonucleotide ClC3bs 5′-CGGGATCCTGCAAGGAACTCATTATACAATG-3′ corresponded to bases 479–500 of the ClC-3 cDNA sequence [17] fused to an *Bam*HI restriction site. Oligonucleotide ClC3xa 5′-GCTCTAGACCTCATCTACAGGACTCAGTTG-3′ corresponded to bases 2796–2775 fused to an *Xba*I site. The cDNA was amplified in a 35-cycle PCR reaction (45 s at 94°C–45 s at 50°C–420 s at 72°C) using Pfu-polymer-

ase. The amplified PCR product of 2318 bp length was purified by agarose gel electrophoresis and ligated into the *Srf*I site of pPCRscript. The *Bam*HI and *Xba*I fragment of ClC-3 was then subcloned into the *Bam*HI/*Xba*I digested oocyte expression vector pGEM-He-Juel [24]. The ClC-3 cDNA was sequenced using the dye termination cycle method to exclude any substitutions possibly caused by PCR amplification. The sequence was found to be identical to the sequence published by Kawasaki et al. [17] except for one polymorphism found in two independent PCR products. This substitution of G by A at position 2171 causes an amino acid exchange from valine to isoleucine at position 716.

A plasmid containing rat ClC-2 was kindly supplied by Prof. T. Jentsch (ZMNH, University of Hamburg, Germany). It consists of the *Xho*I–*Eco*RI fragment of ClC-2 cDNA cloned into the vector pBluescriptSK containing the 5′-UTR of the *Torpedo* ClC-0 cDNA. The *Hin*dIII–*Xho*I fragment of canine I_{Cln} cDNA [13] was excised from the vector pcDNA3.1 and cloned into the same sites of pBluescriptKS.

2.2.4. cRNA expression in X. laevis oocytes

For in vitro transcription, plasmid DNA was linearized with *Sal*I (ClC-3), *Bam*HI (ClC-2) or *Xho*I (I_{Cln}) and transcribed in vitro with T7-RNA polymerase in the presence of a cap analog. The protocol supplied with the polymerase was followed with the exception that all nucleotides and the cap analog were used at two-fold concentrations (1 mM) to increase the yield of complementary RNA (cRNA). Template plasmids were removed by digestion with RNase-free DNase I. The cRNA was purified by phenol/chloroform extraction followed by precipitation with 1/2 vols. of 7.5 M ammonium acetate and 2 vols. of ethanol to remove unincorporated nucleotides. After determination of the amount of cRNA by measuring absorption at 260 nm, integrity of the transcript was verified by denaturing agarose gel electrophoresis.

X. laevis females were obtained from Knysna (South Africa). Oocytes (stages V and VI) were isolated by collagenase treatment of ovary lobes as described before [25] and allowed to recover overnight. They were microinjected with 25–50 nl cRNA in water at a concentration of 0.3–0.6 μg/μl using a nanoliter injector (WPI, Berlin, Germany).

2.2.5. Determination of the solute-accessible oocyte volume

The solute-accessible volume of oocytes was determined by silicon-oil centrifugation. Oocytes were preincubated for 20 min in isotonic ND96 buffer containing 370 kBq ^3H_2O/ml. Then the buffer was diluted to 50% osmolarity with water containing the same amount of ^3H_2O. After incubation of 0–45 min single oocytes were placed on top of a layer of silicon oil (PN200, Roth, Karslruhe, Germany) in a 400 μl microcentrifuge tube. The tube was immediately centrifuged in a Beckmann Microfuge E for 30 s. For the determination of the cell-associated radioactivity, the oocyte-containing tip was cut off with a hot scalpel. The tip was placed upside down in a microcentrifuge tube and the content was extruded by centrifugation. The pellet was homogenized by addition of 750 μl water and vigorous vortexing. The radioactivity was determined by liquid scintillation counting.

2.2.6. Efflux experiments

Oocytes were injected with 50 nl of HEPES-buffered [^{3}H]taurine (24 kBq/μl, 100 mM), Na^{36}Cl (0.925 kBq/μl, 115 mM), [^{3}H]inulin (37 kBq/μl, 1.3 mM) or [^{14}C]lactate (3.7 kBq/μl, 100 mM). Oocytes were allowed to recover for 5–30 min, before the efflux experiment was conducted. Five oocytes were washed two times with 3 ml ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4, 210 mosM) and subsequently suspended in 1 ml ND96 (isotonic conditions) or ND48 (hypotonic conditions, 105 mosM, NaCl reduced to 48 mM). In the case of ^{36}chloride efflux experiments, extracellular sodium chloride was replaced by sodium gluconate. In the experiments in which efflux was elicited by streptolysin S (5000 U/ml) the volume of transport buffer was reduced to 200 μl per group of five oocytes. Over a period of 45 min, aliquot samples of 75 or 15 μl (in the case of streptolysin S) were taken from the supernatant and their radioactivity was determined by liquid scintillation counting. To calculate the efflux curve, the amount of radioactivity in the supernatant at each time point was integrated over time. The released

radioactivity represents the summarized efflux activity of five oocytes per experiment. Therefore, no standard deviations are given. For the figures the released radioactivity was divided by the number of oocytes in the test tube. As no difference in efflux characteristics was found between non-injected and H_2O-injected oocytes, the former were used as controls in the efflux experiments. The efflux measurements were performed 3–6 days after cRNA-injection. Experiments performed with the same batch, but on different days, always resulted in qualitatively comparable data. All experiments were repeated with two to three batches of oocytes. Although efflux rates varied between batches, qualitatively comparable data were obtained. The figures show single representative experiments.

2.2.7. Electrophysiology

Two-electrode voltage-clamp recordings were performed 3–6 days after injection at room temperature using a Geneclamp 500 amplifier (Axon Instruments). From a holding potential of −40 mV voltage ramps from −150 to +50 mV were performed. The data were filtered at 100 Hz and recorded with MacLab digital to analog converter and software for data acquisition and analysis (AD Instruments, Castle Hill, Australia). ND96 buffer (as described above) was used as control solution (superfusate). For the isotonic and hypotonic ND48 solutions, NaCl was reduced to 48 mM and osmolarity was adjusted by adding 96 mM glucose for the isotonic control solution. The flow rate of the superfusion was 20 ml/min and a complete exchange of the bath solution was reached within 10 s. The currents given are steady-state currents for the control conditions and maximal stimulation under hypotonic solution after 30 min.

Data are provided as means ± S.E., n represents the number of oocytes investigated. Therefore, throughout the paper, we show experimental data obtained on the same day for each specific set of experiments. All experiments were repeated with at least two to three batches of oocytes; in all repetitions qualitatively similar data were obtained.

3. Results

3.1. Expression of osmolyte channel candidates in rat brain

Cultured astrocytes show a significant release of chloride and taurine under hypo-osmotic conditions, therefore we investigated the mRNA expression of three candidates potentially involved in the osmolyte release in brain cells. The presence of ClC-2, ClC-3 or I_{Cln} mRNA in RNA or mRNA samples isolated from fetal rat brain (E16), adult brain, neuron-rich primary cultures and astroglia-rich primary cultures was determined by RT-PCR. Plasmids containing the full-length cDNA sequences of the three candidates were used as positive controls (Fig. 1). The three candidates were found to be expressed in fetal brain (data not shown), adult brain as well as in cultured neurons and astrocytes (Fig. 1). For ClC-2, at least two splice variants, ClC-2L and ClC-2S, have been described, which are characterized by the presence or absence of exon 20 [23]. In whole brain and in astroglia-rich primary cultures, both splice variants, ClC-2L and ClC-2S, were detectable. For neuron-rich primary cultures, only the larger splice variant, ClC-2L, was found (Fig. 1).

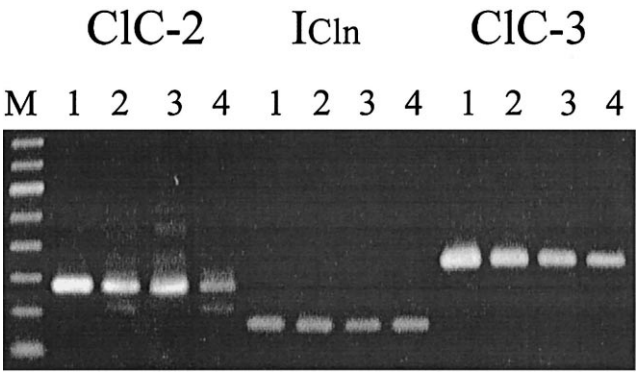

Fig. 1. Expression of ClC-2, ClC-3 and I_{Cln} mRNA in rat brain detected by RT-PCR. The expected size of the amplified products is 275 bp for ClC-2L, 215 bp for ClC-2S, 336 bp for ClC-3 and 177 bp for I_{Cln}. Control reactions were performed with plasmid templates (lane 1). mRNA was isolated from adult rat brain (lane 2) from neuron-rich primary culture (lane 3), or astroglia-rich primary culture (lane 4) and ClC-2, ClC-3 and I_{Cln} were detected subsequently. As DNA-standard, a 100-bp ladder was used (lane M).

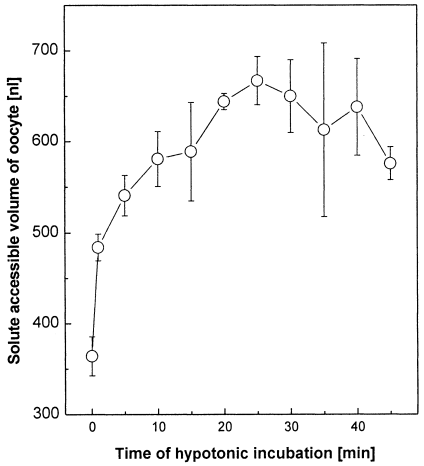

Fig. 2. Swelling of oocytes under hypotonic conditions. The solute accessible volume of the oocytes was calculated from the ^3H_2O content of single oocytes. Oocytes were incubated in ND96 containing ^3H_2O. After reduction of osmolarity by 50% dilution with water (containing the same amount of ^3H_2O), oocytes were removed from the buffer at different times of incubation and stripped of residual buffer by silicon oil centrifugation. Values are means ± S.E.M.

3.2. Volume changes of X. laevis oocytes

In order to study volume regulatory processes in the *Xenopus* oocyte expression system, we first investigated the volume changes of oocytes induced by hypo-osmolarity. *X. laevis* oocytes have a diameter of about 1 mm corresponding to a calculated spherical volume of 525 nl. As the oocytes contain a high percentage of lipid-rich egg yolk, the solute accessible volume is likely to be less than the total volume. We determined the solute accessible volume of the oocytes by equilibration with ^3H_2O-containing buffer. The mean solute-accessible volume of the oocytes was 364 ± 21 nl (S.E.M., $n = 3$), which represents about 70% of the calculated volume of 525 nl. Upon reduction of the osmolarity to 50%, the oocytes swelled to a solute accessible volume of 484 ± 15 nl (S.E.M.) within 1 min. A plateau with a maximal solute accessible volume of 667 ± 27 nl (S.E.M.) was reached after 25 min, which represents an increase to 183% of the volume under isotonic conditions (Fig. 2).

3.3. Efflux experiments

We found oocytes to be a suitable expression system for the investigation of taurine transport. Control oocytes were almost impermeable to taurine. Even after preloading overnight only small amounts of labeled taurine were taken up by the oocytes. Therefore oocytes were preloaded by injection of [^{3}H]taurine. To determine whether this method is suitable for efflux studies, we examined the efflux of lactate through the well-characterized rat H^+/monocarboxylate cotransporter MCT1 [22]. About 55% of injected lactate (5 nmol) was released by MCT1-expressing oocytes under isotonic conditions, the major efflux occurring within the first 20 min. Control oocytes released less than 3% after 1 h of incubation (Fig. 3).

To ensure that the injected taurine was not degraded or irreversibly bound by oocyte components, we studied the efflux of taurine through a pore of less than 3 nm formed by the bacterial toxin streptolysin S [26]. During an incubation of oocytes with streptolysin S (5000 U/ml) for 45 min under isotonic conditions about 60% of the injected taurine was released (Fig. 4), a similar fraction as the released lactate in the experiment described above. On the other hand, only 1% of injected inulin, which is too large to pass through the pores, was released under identical conditions.

3.4. Chloride channel activity

Both ClC-2 and ClC-3 have been described as vol-

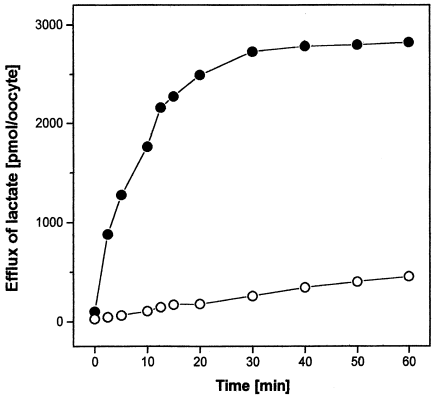

Fig. 3. Efflux of injected [^{14}C]lactate from oocytes. Oocytes were injected with [^{14}C]lactate and were then incubated ND96 in groups of five. At the indicated times of incubation, samples were taken from the supernatant and radioactivity was determined. ●, Oocytes expressing the rat monocarboxylate transporter 1 (MCT1); ○, control oocytes.

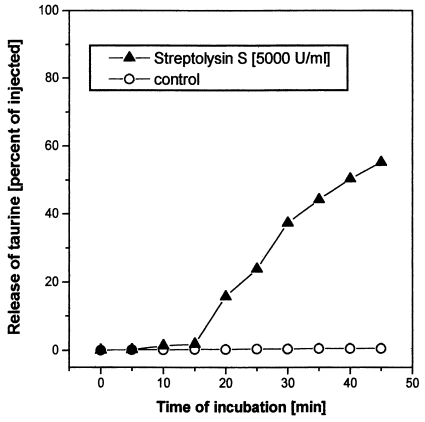

Fig. 4. Efflux of injected [^{3}H]taurine from oocytes through pores formed by streptolysin S. Oocytes were injected with [^{3}H]taurine and were then incubated in groups of five. At the indicated times of incubation, samples were taken from the supernatant and radioactivity was determined. The efflux of untreated control oocytes (○) was compared to the efflux of oocytes treated with 5000 U/ml streptolysin S (▲).

ume-regulated chloride channels [11,12]. I_{Cln} cRNA was shown to induce chloride currents when expressed in oocytes under certain conditions. Therefore, we first studied the chloride channel activity in oocytes expressing these three candidates by electrophysiological and Cl^- efflux experiments.

Following an expression period of 3–6 days, oocytes were injected with $Na^{36}Cl$. After a washing step, the combined efflux of chloride of a group of five oocytes was monitored by removal of aliquots from the isotonic or hypotonic buffer. Comparable results were obtained from at least three different batches of oocytes. Chloride efflux within 45 min varied in control oocytes between 0.2 and 1 nmol per oocyte under isotonic conditions and between 0.6 and 2.7 nmol under hypotonic conditions. In ClC-3 or I_{Cln}-expressing oocytes, no significantly different efflux rates were observed. Under isotonic conditions, the efflux was between 0.2 an 1 nmol which increased under hypotonic conditions only to 0.8–2 nmol. However, in agreement with previously published results, we detected significant release of ^{36}Cl in ClC-2-expressing oocytes, even under isotonic conditions. In a representative experiment, we measured a chloride efflux of about 4 nmol/45 min per oocyte as compared to an efflux of less than 1 nmol/45 min in control oocytes. Reduction of osmolarity to 50% (210 to 105 mosM) increased the efflux after about 20 min significantly. After a 45-min incubation, the efflux was about three-fold (11 nmol/oocyte) increased compared to the efflux under isotonic conditions (Fig. 5). When compared to the course of swelling (Fig. 2), it seemed that the opening of ClC-2 requires a significant increase of oocyte volume to about twice the original volume.

The I_{Cln} protein has also been proposed to act as a regulator of ClC-3 [20]. However, coexpression of ClC-3 and I_{Cln} cRNA did not lead to an increased efflux of chloride, neither under isotonic nor under hypotonic conditions when compared to oocytes expressing ClC-3 or I_{Cln} alone (Fig. 5). The coexpression of ClC-2 with ClC-3 similarly did not result in any significant increase of ^{36}Cl release when compared to oocytes expressing ClC-2. These results were confirmed by electrophysiological determination of chloride conductance.

ClC-3-, I_{Cln}- or ClC-3+I_{Cln}-expressing oocytes showed conductances below 4 μS and were not sig-

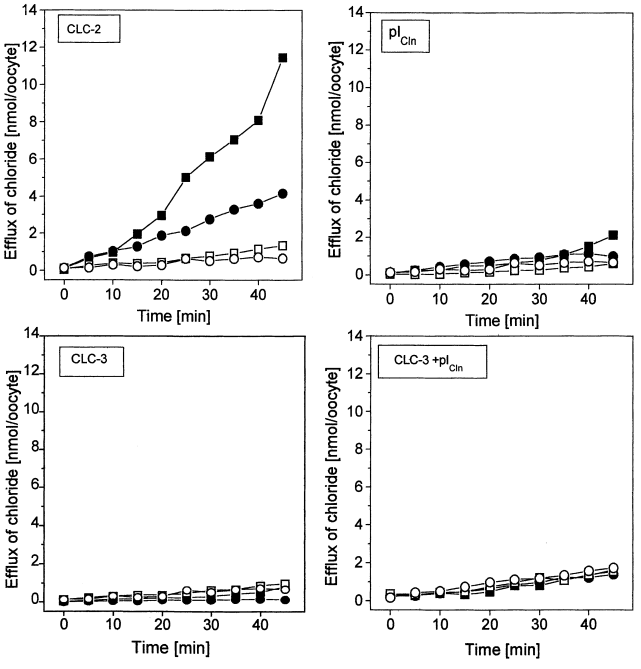

Fig. 5. Efflux of chloride from oocytes expressing ClC-2, I_{Cln}, ClC-3 and ClC-3+I_{Cln}. Oocytes expressing ClC-2, I_{Cln}, ClC-3 or ClC-3+I_{Cln} (solid symbols in each figure) or control oocytes (open symbols) were injected with $Na^{36}Cl$ and were then incubated in isotonic or hypotonic buffer in groups of five oocytes. At the indicated times of incubation, samples were taken from the isotonic (○, ●) or hypotonic (□, ■) incubation buffer and radioactivity was determined.

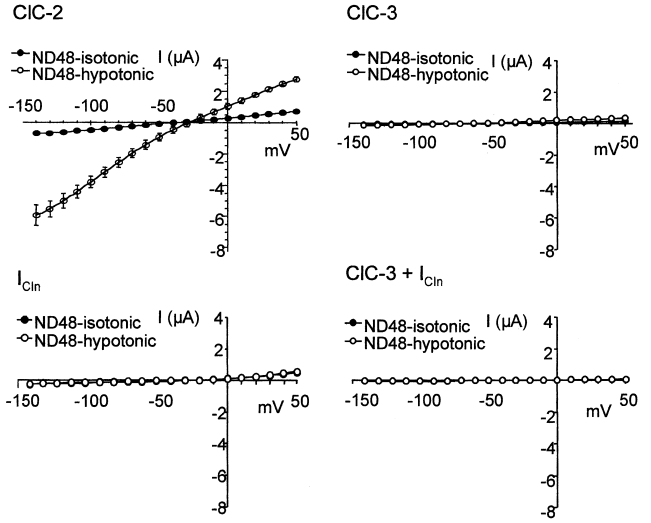

Fig. 6. Current–voltage relation in ClC-2-, I_{Cln}-, ClC-3- and ClC-3+I_{Cln}-expressing oocytes. Oocytes were injected with cRNAs of ClC-2, I_{Cln}, ClC-3 or ClC-3+I_{Cln}. Voltage ramps were performed between −150 and +50 mV under isotonic (○) and hypotonic conditions (●).

nificantly different from control oocytes under isotonic or hypotonic conditions as determined in several batches of oocytes (Fig. 6). ClC-2-expressing oocytes, in contrast, showed 8.47 ± 1.3 μS ($n = 7$) conductance which increased within 20 min of hypotonic incubation to 35.4 ± 3.5 μS ($n = 7$) (Fig. 6).

3.5. Osmolyte channel activity

To test whether any of the candidates were able to mediate the efflux of taurine, we performed efflux experiments with [^{3}H]taurine-injected oocytes. Variations of taurine efflux rates were found between oocyte batches. Minimum efflux rates of taurine under hypotonic conditions were 170 pmol/45 min (per oocyte) in cRNA-injected oocytes and 40 pmol/45 min in the control. Whereas, in a different batch, maximum taurine efflux rates of 840 pmol/45 min and 315 pmol/45 min were measured in cRNA-injected and in controls, respectively. However, there was always a significant difference between control oocytes and cRNA-injected oocytes as well as between isotonic and hypotonic conditions. In the representative experiments depicted in Fig. 7, the basal taurine efflux in control oocytes varied between 15 and 40 pmol per oocyte within 45 min under isotonic conditions. Under hypotonic conditions efflux varied between 94 and 315 pmol per oocyte within 45 min. Under isotonic conditions the taurine efflux rate in oocytes injected with cRNA coding for ClC-2, ClC-3 or I_{Cln} were similar to the efflux of control oocytes, varying between 18 and 166 pmol of taurine within 45 min per oocyte. However, under hypotonic conditions, a significantly increased taurine efflux between 700 and 840 pmol per oocyte within 45 min was observed for each of the expressed cRNAs (Fig. 7). The hypotonicity induced efflux of taurine followed a similar time course as the release of chloride by hypotonicity activated ClC-2, showing a lag phase of about 10–20 min. After 45 min, about 20% of the injected taurine was released from the oocytes.

In order to find out whether this taurine efflux represents a cRNA-specific or an endogenous response, we performed the same experiment with MCT1-expressing oocytes. Surprisingly, we also observed a swelling-induced efflux of taurine of about 700 pmol/45 min per oocyte in the MCT1-expressing

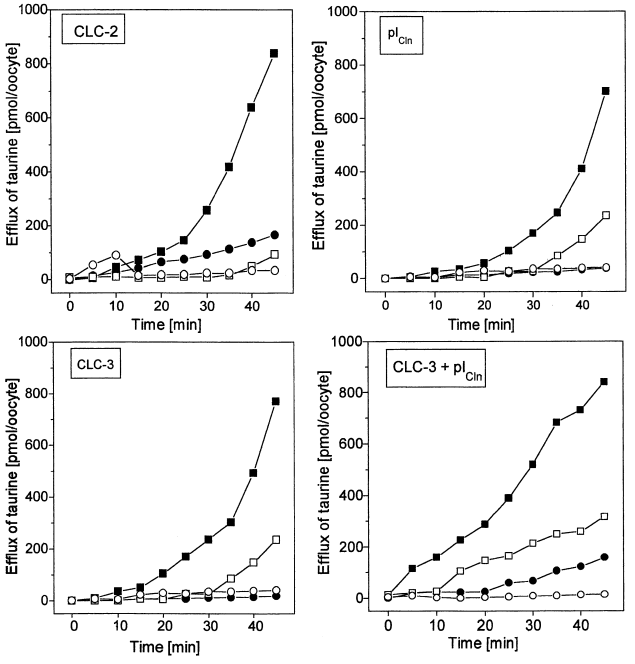

Fig. 7. Efflux of taurine from oocytes expressing in ClC-2, I_{Cln}, ClC-3 and ClC-3+I_{Cln}. Oocytes expressing ClC-2, I_{Cln}, ClC-3 or ClC-3+I_{Cln} (solid symbols in each figure) or control oocytes (open symbols) were injected with [^{3}H]taurine and were then incubated in isotonic or hypotonic buffer in groups of five oocytes. At the indicated times of incubation, samples were taken from the isotonic (○, ●) or hypotonic (□, ■) incubation buffer and radioactivity was determined.

oocytes (data not shown). Control oocytes showed an efflux of about 250 pmol/45 min under hypotonic conditions (data not shown).

Overexpression of membrane proteins might have caused unspecific leakage of the substrate in these experiments. Therefore, we examined the permeability of non-injected and ClC-3-expressing oocytes for [^{3}H]inulin under isotonic and hypotonic conditions. Only a marginal efflux of inulin was observed (less than 0.5%), irrespective of the osmolarity of the buffer.

4. Discussion

Several channels have been discussed as pathways of organic osmolyte efflux. In this study, we tested two candidates of the ClC-family of chloride channels, ClC-2 and ClC-3, and the putative osmolyte channel protein I_{Cln} for the efflux of chloride and taurine. These proteins were chosen because they are the most probable candidates for osmolyte channels and are all expressed in astrocytes, a cell type which is known to release taurine during hypo-osmotic stress [1].

Rat ClC-2 has been expressed in *Xenopus* oocytes and is known to be volume-sensitive [11]. In our study, we were able to demonstrate the volume-sensitivity of ClC-2 by electrophysiological recordings and flux studies using the radioactive isotope $^{36}Cl^-$. An efflux of about 20 nmol Cl^-/h was detected. This would correspond to a current of about 0.5 μA. At the resting potential of oocytes, which lies close to −40 mV, currents within a similar range were determined under voltage-clamp conditions.

Both ClC-3 and I_{Cln} have been reported to act as chloride channels when expressed in oocytes [13,17]. However, subsequent experiments in other laboratories failed to demonstrate chloride channel activity with ClC-3. Even coexpression of ClC-3 with two other members of the ClC-family, ClC-4 and ClC-5, did not result in any change of the channel characteristics of single ClC-5 [27]. Using an epitope-tag construct, we were able to demonstrate a significant expression of ClC-3 in the plasma membrane of oocytes under our conditions (unpublished result). The function of the I_{Cln} protein as an osmolyte channel is a matter of debate. Expression cloning suggests that I_{Cln} itself constitutes a chloride channel [13]. Although the protein was detected in the cytosol under isotonic conditions, hypotonic shock results in the association with the membrane fraction [28]. In contrast, it was shown that expression of ClC-6 in *X. laevis* oocytes results in the induction of similar currents as those reported for I_{Cln} and it was concluded that the expression of these proteins might upregulate endogenous oocytes channels [29]. However, the expression was very low and had to be optimized by an increase of the incubation temperature [29]. It has been further proposed that the I_{Cln} protein might act as an activator of ClC-3 [20]. In our experiments, however, coexpression of both proteins did not result in the expression of significant chloride fluxes. To study taurine release, we have used collagenase-treated oocytes, which have a low level of oocyte endogenous transporters and have been successfully used in the expression of a broad variety of transporters and channels (amino acid transporters, monocarboxylate transporters, non-specific anion transporters, sodium channels). In agreement with this notion, we could not detect the oocyte endogenous volume-regulated chloride channel, which reportedly can only be detected in manually defolliculated oocytes shortly after the manipulation, but not in collagenase-treated oocytes [30,31]. In these oocytes, we could not detect I_{Cln}-induced chloride currents, which might result from a failing association of the I_{Cln} protein to the plasma membrane under hypotonic conditions or the enzmyatic removal of the follicular cell layer. To our surprise, however, we detected significant volume-sensitive taurine efflux in oocytes expressing ClC-2, ClC-3, I_{Cln} and MCT1. This efflux was not caused by unspecific leakage of the oocytes, since we could show that injected labeled inulin was not released under these conditions. Two alternative conclusions are compatible to this observation: (1) all four proteins mediate the efflux of taurine irrespective of any Cl^- channel activity; and (2) overexpression of these heterologous membrane proteins results in the upregulation of an endogenous taurine efflux pathway in *Xenopus* oocytes. Considering the unrelatedness of the four transport molecules, the latter conclusion seems more likely. The second conclusion is also supported by an upregulation of taurine efflux by expression of the membrane protein h4F2hc, which under physio-

logical conditions mediates the plasma membrane trafficking of amino acid transporters [32].

It has been proposed by different groups [1,2,33] that volume-sensitive osmolyte release is mediated by non-specific anion channels. However, taurine-specific pathways have been proposed as well [5,9,34]. The experiments presented here, clearly show an induction of taurine release in oocytes which does not coincide with a significant increase in chloride permeability. Thus the expression of I_{Cln}, ClC-2, ClC-3, or MCT1 is likely to upregulate an oocyte endogenous osmolyte channel with these properties.

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